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(71) Applicant *(for all designated States except US):* NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK).

(72) Inventors; and
(75) Inventors/Applicants *(for US only) :* DAMHUS, Ture [DK/DK]; Livjaegergade 43 st. tv., DK-2100 Copenhagen Ø (DK). NIELSEN, Egon [DK/DK]; Niels W. Gades Gade 33 st., DK-2100 Copenhagen Ø (DK). ÄSLYNG, Dorrit, Anita [DK/DK]; Fyrren 8, Svogerslev, DK-4000 Roskilde (DK).

(74) Agent: NOVO NORDISK A/S; Patent Department, PeV, Novo Allé, DK-2880 Bagsvaerd (DK).

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(57) Abstract

The present invention relates to the use of proteases derived from members of the genus *Nocardiopsis* in detergent additives or compositions, or wash liquors, comprising specific bleaching systems.

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DETERGENT COMPOSITIONS

TECHNICAL FIELD

The present invention relates to the use of proteases derived from members of the genus *Nocardiopsis* in detergent additives or compositions, or wash liquors, comprising specific bleaching systems.

BACKGROUND ART

Bleaching systems have been suggested for incorporation into detergent compositions in order to obtain bleaching effects on stained fabric, or in order to prevent transfer of a textile dye from a dyed fabric to another fabric during washing or rinsing.

Detergent compositions or wash liquors comprising a bleaching system have been described in e.g. International Patent Publications WO 89/09813 and WO 91/05839. Bleaching systems as described herein comprise enzymes exhibiting peroxidase activity and hydrogen peroxide or a precursor thereof, or enzymes exhibiting a suitable oxidase activity.

A major drawback in applying such bleaching systems to detergent compositions is that proteases present in such compositions may be strongly affected by the bleaching systems, thereby hampering the washing performance of the detergent composition.

Some members of the genus *Nocardiopsis* are known to produce proteases. In International Patent Application WO 88/03947, alkaline proteases obtainable from protease producing strains of *Nocardiopsis* have been described for their use as detergent additives, in particular as detergent additives for cold water laundering.

SUMMARY OF THE INVENTION

We have now surprisingly found that proteases derived from members of the genus *Nocardiopsis* are more stable in the presence of the above mentioned bleaching systems than other detergent proteases.

Accordingly, the present invention provides a detergent composition comprising a protease derived from a member of the genus *Nocardiopsis*, and: (a) an enzyme exhibiting peroxidase activity and hydrogen peroxide or a precursor thereof, and/or (b) an enzyme exhibiting a suitable oxidase activity.

In another aspect, the invention provides a detergent additive comprising a protease derived from a member of the genus *Nocardiopsis*, and: (a) an enzyme exhibiting peroxidase activity and hydrogen peroxide or a precursor thereof, and/or (b) an enzyme exhibiting a suitable oxidase activity.

DETAILED DISCLOSURE OF THE INVENTION

The present invention relates to proteases derived from members of the genus *Nocardiopsis*, which proteases according to the invention have proved to be stable in the presence of peroxidase based bleaching systems.

More specifically, the invention relates to the use of proteases derived from members of the genus *Nocardiopsis* in cleaning processes, e.g. household laundering, industrial and institutional laundering or cleaning, and dish washing, or fabric cleaning processes, in which processes solutions containing enzymes exhibiting peroxidase activity, or enzymes exhibiting suitable oxidase activity, are used for the purpose of either bleaching stains on surfaces in contact with the solutions, or inhibiting the transfer of a textile dye from a dyed fabric to another fabric.

The present invention provides detergent compositions comprising proteases derived from a member of the genus *Nocardiopsis*, and enzymes exhibiting peroxidase activity together with hydrogen peroxide or a precursor thereof, or alternatively enzymes exhibiting a suitable oxidase activity.

The invention also provides detergent additives comprising proteases derived from a member of the genus *Nocardiopsis*, and enzymes exhibiting peroxidase activity together with hydrogen peroxide or a precursor thereof, or alternatively enzymes exhibiting a suitable oxidase activity.

Optionally, the detergent additive or detergent composition also contains accelerators.

Nocardiopsis Proteases

Microorganisms belonging to the actinomycete *Nocardiopsis* are well known in the literature. Some examples of species and strains described are *N. dassonvillei*, Type Strain ATCC 23218; *N. dassonvillei* M58-1 (NRRL 18133), WO Pat. Publ. 88/03947; *N. dassonvillei* ZIMET 43647, DD Pat. Publ. 200,432; *N. dassonvillei* subsp. prasina, Agric.Biol.Chem. (54, 8, 2177-79) 1990; *N. sp.* OPC 120, JP Pat. Appl. 2,255,081; and *N. sp.* 10R (NRRL 18262), WO Pat. Publ. 88/03947.

Proteases derived from members of the actinomycete *Nocardiopsis* are disclosed in e.g. International Patent Application WO 88/03947 and GDR Patent No. DD 200,432. Proteases obtainable from the *Nocardiopsis* are alkaline proteases.

Preferably, the proteases are derived from a protease producing strain of *N. dassonvillei*, preferably the strain ZIMET 43647, more preferred the strain *N. dassonvillei* M58-1 (NRRL 18133), or from a protease producing strain of the species defined by the strain 10R, more preferred the strain *Nocardiopsis sp.* 10R (NRRL 18262).

The strains *N. dassonvillei* M58-1 and *Nocardiopsis sp.* 10R are described in the above mentioned International Patent Application WO 88/03947, and accordingly have been deposited under the terms of the Budapest Treaty, at the Agricultural Research Culture Collection (NRRL), Peoria, US (NRRL 18133 was deposited on 1986.11.13; NRRL 18262 was deposited on 1987.11.10).

The strain ZIMET 43647 is described in the above mentioned DD Patent No. 200,432.

In a more preferred embodiment, proteases are derived from a protease producing strain of *Nocardiopsis* that is characterized by having optimal pH for growth at about pH 9, by having essentially no growth below pH 8, by having

optimal temperature for growth at 20-30°C, by essentially no growth above 35°C, and by belonging to *N. dassonvillei*, preferably *N. dassonvillei* M58-1 (NRRL 18133), or the strain ZIMET 43647, or to the species defined by the strain 10R, preferably *Nocardiopsis sp.* 10R (NRRL 18262).

In another preferred embodiment, the protease is an alkaline protease preparation derived from *Nocardiopsis*, preferably a strain of *N. dassonvillei*, more preferred the strain *N. dassonvillei* M58-1 (NRRL 18133), or to the species defined by the strain 10R, preferably *Nocardiopsis sp.* 10R (NRRL 18262), characterized by having at least 60% of its maximum activity in the pH range of from pH 7 to 11, measured with casein as substrate.

Suitable protease dosages may be in the range 0.0001 to 10 mg of enzyme protein per litre of washing liquor, preferably 0.001 to 1 mg of enzyme protein per litre of washing liquor. In detergent compositions, suitable protease dosages may be in the range of 0.005 μg to 30 mg of enzyme protein per g of detergent composition, preferably 0.05 μg to 3 mg of enzyme protein per g of detergent composition, more preferred 0.1 μg to 100 μg of enzyme protein per g of detergent composition.

Enzyme Exhibiting Peroxidase Activity

Enzymes exhibiting peroxidase activity are understood to indicate enzymes with a mode of action similar to that of a peroxidase (EC 1.11.1.7; according to the Recommendations of the Nomenclature Committee of the International Union of Biochemistry), and will be used synonymously therewith.

Peroxidases suitable for incorporation into detergent additives or compositions of the invention have been described in e.g. the previously mentioned International Patent Application Nos. WO 89/09813 and WO 91/05839, which peroxidases are hereby incorporated by reference.

Peroxidases to be employed for the present purpose may be isolated from and are producible by plants (e.g. horseradish peroxidase), or microorganisms, particularly bacteria or fungi, e.g actinomycetes or basidiomycetes, preferably derived from a strain of *Coprinus*, preferably *C. cinereus*.

Other useful peroxidases are haloperoxidases such as chloro or bromo peroxidases.

Peroxidases may also be producible by methods comprising cultivating a host cell transformed with a recombinant DNA vector carrying a DNA sequence encoding said enzyme as well as DNA sequences encoding functions permitting the expression of the enzyme, in a culture medium under conditions permitting the expression of the enzyme and recovering the enzyme from the culture.

Preferably, the peroxidase is active at in the range of pH 6.5 to 12, more preferred pH 6.5 to 10.5, and most preferred pH 7.5 to 10.5.

Suitable peroxidase dosages may be in the range of 0.01 to 100 mg/l of wash liquor, more preferred 0.1 to 10 mg/l, most preferred 0.1 to 1 mg/l. In detergent compositions, suitable peroxidase dosages may be in the range of 0.5 μg to 300 mg enzyme protein per g of detergent composition, preferably 5 μg to 30 mg of enzyme protein per g of detergent composition, more preferred 50 μg to 3 mg of enzyme protein per g of detergent composition.

Hydrogen Peroxide or Precursors

When the enzyme used in the bleaching system is a peroxidase, hydrogen peroxide or a precursor of hydrogen peroxide, preferably perborate or percarbonate, will typically be added. It may, however, be desirable to utilize an enzymatic process for the formation of hydrogen peroxide.

One such category of hydrogen peroxide generating systems comprises enzymes which are able to convert molecular oxygen and an organic or inorganic substrate into hydrogen peroxide and the oxidized substrate, respectively.

Preferred hydrogen peroxide-generating enzymes are those which act on cheap and readily available substrates which may conveniently be included into detergent additives or compositions. An example of such a substrate is glucose which may be utilized for hydrogen peroxide production by means of glucose oxidase. Other suitable oxidases are urate oxidase, galactose oxidase, alcohol oxidases, amine oxidases, amino acid oxidase, and cholesterol oxidase.

Optimal hydrogen peroxide concentrations in wash liquors are within the range of 1 μM to 20 mM, preferably 1 μM to 1 mM. When using *Coprinus* peroxidase, 0.01 to 0.25 mM hydrogen peroxide is preferred.

Enzymes Exhibiting Oxidase Activity

In the context of this invention, enzymes exhibiting oxidase activity are understood to indicate enzymes with a similar mode of action to that of an oxidase, and are meant to be synonymous therewith in the following.

Examples of enzymes exhibiting a suitable oxidase activity are oxidases which act on aromatic compounds, in particular phenolic, e.g. polyphenolic, are catechol oxidase (EC 1.10.3.1) or laccase (EC 1.10.3.2).

Accelerators

It has been found that the addition of certain oxidizable substances at the beginning of, or during the washing and/or rinsing process, may enhance the dye transfer inhibitory effect of the peroxidase system employed. Such substances are termed enhancers or accelerators, since they generally increase the initial rate of the reaction between peroxidase/hydrogen peroxide and textile dyes.

Examples of potential accelerators are metal ions, e.g. Mn^{++}, halide ions, e.g. chloride or bromide ions, or organic compounds such as phenols, e.g. p-hydroxybenzoic acid, p-hydroxycinnamic acid, 2,4-dichlorophenol, p-hydroxybenzenesulfonic acid, 7-hydroxycoumarin, or vanillin, or those given in M. Kato and S. Shimizu, *Plant Cell Physiol.* 26(7), 1985, pp. 1291-1301 (cf. Table 1 in particular) or in B.C. Saunders et al., *op. cit.*, p. 141 ff.

Optimal accelerator concentration in wash liquors is within the range of 1μM to 1mM, preferably 5 to 100 μM.

Detergent Additives And Detergent Compositions

The detergent composition of the invention may comprise one or more surfactants which may be of an anionic, non-ionic, cat-ionic, amphoteric or zwitter-ionic type, or a mixture of these. Typical examples of anionic surfactants are linear alkyl benzene sulfonates (LAS); alkyl sulfates (AS); alpha olefin sulfonates (AOS);

alcohol ethoxy sulfates (AES) and alkali metal salts of natural fatty acids. Examples of non-ionic surfactants are alkyl polyethylene glycol ethers; nonylphenol polyethylene glycol ethers; fatty acids esters of sucrose and glucose; and esters of polyethoxylated alkyl glucoside.

The detergent composition of the invention may also contain other detergent ingredients known in the art such as builders, anti-corrosion agents, sequestering agents, anti soil-redeposition agents, perfumes, stabilizers for the enzymes and bleaching agents, formulations aids, optical brighteners, foam boosters, chelating agents, fillers, fabric softeners, etc. The detergent composition of the invention may be formulated substantially as described in *Falbe, J.*; Surfactants in Consumer Products. Theory, Technology and Application; Springer Verlag 1987, *vide* in particular the section entitled "Frame formulations for liquid/powder heavy-duty detergents".

The detergent compositions of the invention can be formulated in any convenient form such as powders, liquids, etc. Generally, detergent compositions are used in dosages within the range of 0.3 to 15 g of detergent per litre of wash liquor.

The detergent composition of the invention may advantageously include one or more other enzymes, e.g. lipases, amylases, cellulases, conventionally included in detergent compositions, as well as proteases of other origin.

The enzymes according to the invention may be included in a detergent composition by adding separate additives containing one or more enzymes, or by adding a combined additive comprising all of these enzymes.

The additive of the invention, whether being a separated additive or a combined additive, can be formulated e.g. as granulates, liquids, slurries, etc. Preferred detergent additive formulations are non-dusting granulates, liquids, in particular stabilized liquids, slurries, or protected enzymes. Dust free granulates may be produced according to e.g. GB Patent No. 1,362,365 or US Patent No. 4,106,991, and may optionally be coated by methods known in the art. The enzymes may be mixed before or after granulation. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as e.g. propylene glycol; a sugar or sugar alcohol; lactic acid or boric acid, according to established methods. Other enzyme

stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP Patent Application No. 238,216.

The following example further illustrates the present invention, and is not intended to be in any way limiting to the scope of the invention as claimed.

EXAMPLE

Wash Performance

This example illustrates protease wash performance in the presence of an accelerated peroxidase system in comparison with the wash performance in the absence of this peroxidase system.

The wash performance tests were accomplished on grass juice soiled cotton at 35°C, isothermically for 15 minutes.

1 g/l of a commercial American type phosphate-based powder detergent without bleach was used. The detergent was dissolved in approximately 6° dH (German hardness) water. pH in the wash liquor was 8.5. The textile/wash liquor ratio was approximately 3.5 g of textile (2.3 g of soiled and 1.2 g of clean textile) per litre of detergent solution.

Proteases were dosed to 0, 0.3, and 0.5 mg of enzyme protein per litre. The protease preparation was obtained from *Nocardiopsis sp.* 10R according to International Patent Publication WO 88/03947, which publication is hereby included by reference.

In one set of tests peroxidase 0.4 mg/l, 50 μM sodium p-hydroxybenzenesulfonate (as accelerator), and 0.2 mM H_2O_2 (in the Tables below collectively referred to as the POD-system), and protease were added to the detergent solution prior to addition of soiled textile. The peroxidase used was derived from *Coprinus cinereus*, and obtained according to the method described in pending EP Patent Application No. 91610022.

In another set of tests only the protease was added to the detergent solution prior to addition of soiled textile.

Subsequent to washing, the fabric was rinsed in running tap water and air-dried. The protease performance was determined by the change (ΔR) of the remission (%R) at 460 nm measured on a Datacolor Elrephometer 2000, ΔR being the remission after wash with protease added minus the remission after wash with no protease added.

The results of these comparative tests are presented in Tables 1 and 2 below.

Table 1

Wash performance (ΔR) in the presence and in the absence of the POD-system.

	POD-system present		POD-system absent	
Protease Dosage (mg/l)	0.3	0.5	0.3	0.5
Nocardiopsis protease	8.5	12.4	11.7	15.4
Alcalase™[1)]	0.4	0.7	17.3	18.8
Savinase™[1)]	2.8	4.4	9.3	11.6
Durazym™[1)]	3.6	4.5	13.8	16.0

[1)] Alcalase™, Savinase™, and Durazym™ are trademarks for commercial detergent proteases, supplied by Novo Nordisk A/S, Denmark. The proteases are all alkaline Bacillus proteases.

From Table 1 it appears that the Nocardiopsis protease is significantly less affected by the presence of the bleaching system than are the Bacillus proteases.

These results are also illustrated by Table 2 below. In this table, the stability of the Nocardiopsis proteases is presented as the wash performance of the proteases in the presence of the bleaching system relative to the corresponding performance in the absence of this system.

Table 2

Wash performance in the presence of the POD-system, relative to the wash performance in the absence of the POD-system.

Protease Dosage (mg/l)	% Wash Performance 0.3	0.5
Nocardiopsis protease	73	81
Alcalase™	2	4
Savinase™	30	38
Durazym™	26	28

From Table 2 it appears that in the presence of the bleaching system the Nocardiopsis protease maintains approximately 3/4 or more of its wash performance in the absence of this bleaching system, whereas the Bacillus proteases loose most of their wash performance in the presence of the bleaching system.

CLAIMS

1. A detergent composition comprising a protease derived from a member of the genus *Nocardiopsis*, and

(a) an enzyme exhibiting peroxidase activity and hydrogen peroxide or a precursor thereof, and/or

(b) an enzyme exhibiting a suitable oxidase activity.

2. A detergent composition of claim 1, comprising a protease derived from a protease producing strain of *N. dassonvillei*, or from a protease producing strain of the species defined by the strain 10R.

3. A detergent composition of claim 2, in which the protease is derived from a protease producing strain of *N. dassonvillei*, further characterized by having optimal pH for growth at about pH 9, by having essentially no growth below pH 8, by having optimal temperature for growth at 20-30°C, by essentially no growth above 35°C.

4. A detergent composition of either of claims 2-3, the protease being derived from the strain *N. dassonvillei* M58-1 (NRRL 18133), or from the strain *Nocardiopsis sp.* 10R (NRRL 18262), or a mutant or a variant thereof.

5. A detergent composition of claim 4, the protease being further characterized by having at least 60% of its maximum activity in the pH range of from pH 7 to 11, measured with casein as substrate.

6. A detergent composition of either of claims 2-3, the protease being derived from the strain ZIMET 43647, or a mutant or a variant thereof.

7. A detergent composition of any of claims 1-6, in which the enzyme exhibiting peroxidase activity is horseradish peroxidase, or a peroxidase derived from a strain of *Coprinus*, preferably *C. cinereus*.

8. A detergent composition of any of claims 1-7, in which the precursor for hydrogen peroxide is perborate or percarbonate.

9. A detergent composition of any of claims 1-8, in which the enzyme exhibiting a suitable oxidase activity is catechol oxidase or laccase.

10. A detergent composition of any of claims 1-9, further comprising an accelerator.

11. A detergent composition of claim 10, in which the accelerator is a metal ion, a halide ion, or an organic compound such as a phenol, e.g. p-hydroxybenzoic acid, p-hydroxycinnamic acid, 2,4-dichlorophenol, p-hydroxybenzenesulfonate, 7-hydroxycoumarin, or vanillin.

12. A detergent composition of any of claims 1-11, which further comprises one or more other enzymes, in particular lipases, amylases, cellulases, as well as proteases of other origin than *Nocardiopsis*.

13. A detergent composition of any of claims 1-12, provided in the form of a powder detergent or in the form of a liquid detergent.

14. A detergent additive comprising a protease derived from a member of the genus *Nocardiopsis*, and

(a) an enzyme exhibiting peroxidase activity and hydrogen peroxide or a precursor thereof, and/or

(b) an enzyme exhibiting a suitable oxidase activity.

15. A detergent additive of claim 14, comprising a protease derived from a protease producing strain of *N. dassonvillei*, or from a protease producing strain of the species defined by the strain 10R.

16. A detergent additive of claim 15, in which the protease is derived from a protease producing strain of *N. dassonvillei*, further characterized by having optimal pH for growth at about pH 9, by having essentially no growth below pH 8, by having optimal temperature for growth at 20-30°C, by essentially no growth above 35°C.

17. A detergent additive of either of claims 15-16, the protease being derived from the strain *N. dassonvillei* M58-1 (NRRL 18133), or from the strain *Nocardiopsis sp.* 10R (NRRL 18262), or a mutant or a variant thereof.

18. A detergent additive of claim 17, the protease being further characterized by having at least 60% of its maximum activity in the pH range of from pH 7 to 11, measured with casein as substrate.

19. A detergent additive of either of claims 15-16, the protease being derived from the strain ZIMET 43647, or a mutant or a variant thereof.

20. A detergent additive of any of claims 14-19, in which the enzyme exhibiting peroxidase activity is horseradish peroxidase, or a peroxidase derived from a strain of *Coprinus*, preferably *C. cinereus*.

21. A detergent additive of any of claims 14-20, in which the precursor for hydrogen peroxide is perborate or percarbonate.

22. A detergent additive of any of claims 14-21, in which the enzyme exhibiting a suitable oxidase activity is catechol oxidase or laccase.

23. A detergent additive of any of claims 14-22, further comprising an accelerator.

24. A detergent additive of claim 23, in which the accelerator is a metal ion, a halide ion, or an organic compound such as a phenol, e.g. p-hydroxybenzoic

acid, p-hydroxycinnamic acid, 2,4-dichlorophenol, p-hydroxybenzenesulfonate, 7-hydroxycoumarin, or vanillin.

25. A detergent additive of any of claims 14-24, which further comprises one or more other enzymes, in particular lipases, amylases, cellulases, as well as proteases of other origin than *Nocardiopsis*.

26. A detergent additive of any of claims 14-25, provided in the form of a granulate, preferably a non-dusting granulate, a liquid, in particular a stabilized liquid, a slurry, or a protected enzyme.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 92/00383

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C11D 3/386
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: C11D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A1, 8803947 (NOVO INDUSTRI A/S), 2 June 1988 (02.06.88), claims 12-18, page 1, line 27 - page 2, line 1-4; page 6, line 4-6; page 11, line 1-26	1-6,14-19
Y	WO, A1, 8909813 (NOVO INDUSTRI A/S), 19 October 1989 (19.10.89), claims 1-4,9-10,12, 13-17; page 5, line 33-35	1-8,12, 14-21,25-26
A	WO, A1, 9105839 (NOVO NORDISK A/S), 2 May 1991 (02.05.91)	1-26

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
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Date of the actual completion of the international search	Date of mailing of the international search report
23 March 1993	29 -04- 1993
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INTERNATIONAL SEARCH REPORT
Information on patent family members

26/02/93

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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 8803947	02/06/88	DE-A-	3779753	16/07/92
		EP-A,B-	0290567	17/11/88
WO-A1- 8909813	19/10/89	AU-B-	617811	05/12/91
		AU-A-	3551989	03/11/89
		DE-U-	6890098	16/04/92
		EP-A,B-	0424398	02/05/91
WO-A1- 9105839	02/05/91	AU-A-	6515790	16/05/91
		AU-A-	6516090	16/05/91
		CA-A-	2067748	14/04/91
		CN-A-	1051600	22/05/91
		EP-A-	0495836	29/07/92